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EDITORIAL

NOT IN THE PROPER REALM OF PHARMACY

TO the outside observer, nothing must seem more incongruous than the sale of alcoholic beverages in otherwise highly professional and well-kept pharmacies. Indeed, even to many of us who are accustomed to seeing all kinds of merchandise displayed in some drugstores, the sight of shelves full of whiskey and related alcoholic beverages causes a certain uneasiness of conscience. This inner disturbance is not necessarily because of any adherence to the principles of the Women's Christian Temperance Union but because alcoholic beverages as compared to drugs and medicines are so diametrically opposed in their effect on human welfare.

While it is true that whiskey and brandy are still recognized in the *National Formulary* as official drugs so also are strychnine, arsenic, and a number of other poisons. There is also much to be said for the legitimate medicinal use of certain alcoholic preparations since, for the aged, they are frequently prescribed and, usually, with desirable results. On the other hand, if one surveys the over-all effect of alcoholic beverages on the moral, spiritual, and physical welfare of people generally, the conclusion must be reached that they exact a terrific toll in human suffering, debauchery, and misery. If it were indeed possible to completely eliminate from the world all alcoholic beverages and do this absolutely, it would be one of the greatest contributions to human welfare ever conceived. It is, of course, not possible to completely eliminate alcohol from the affairs of men. It was tried, and the failure of Prohibition in the United States is accepted by almost everyone, drinkers and non-drinkers alike.

The thing which makes the sale of alcoholic beverages in the pharmacy so out of place is that our profession, like medicine, is presumably dedicated to the furtherance of public health and welfare, and pharmacists are presumed to place the welfare of their customers and their community above every other consideration. It is impossible to sell alcoholic beverages without doing so to many people for whom their use is certain to prove harmful to the extreme.

It is also impossible for the seller to use any discernment concerning those who will use them properly and carefully and those who will use them to the point that both their minds and bodies are destroyed. Surely, one cannot imagine a physician operating a liquor store as a part of his office practice and, yet, pharmacists who sell alcoholic beverages are placed in exactly this same incongruous position.

The fact is sometimes overlooked that intoxication with alcohol is a form of poisoning. It just happens that symptoms of poisoning with this toxic material are considered socially acceptable and even humorous in most quarters, but this does not eliminate the fact that the person is under the influence of a poison. The fact that most people recover, at least in part, from the effects of alcohol still does not alter the case. It must be recognized that thousands die each year from cirrhosis of the liver, malnutrition, and many other long term effects from the misuse of alcohol including serious mental disturbances.

It is not likely that this editorial nor a recent action by the American Association of Colleges of Pharmacy condemning the sale of alcoholic beverages in pharmacies will have much effect on the present practice in those States where this is done. These pharmacists are so involved in this activity as a means of deriving greater profit that they may be expected to raise all sorts of counter arguments rationalizing such sale. Be that as it may, no rational argument which is valid can be raised to justify this practice. Either pharmacy and pharmacists are dedicated to the public welfare, without exception, or they are not. Rationalization, expediency, and evasion of such fundamental points of logic is all too common in our profession, and it is this continuing failure of pharmacy to put its house in order which has caused some lay writers to picture us as a dying profession. To the extent that we contribute to human misery we are.

L. F. Tice

BIOCHEMICAL ASPECTS OF BACTERIAL ENDOSPORE FORMATION AND GERMINATION [1]

By R. L. Stedman,* Sc. D.

Part II
Chemical Changes During Sporulation and Germination

IN Part I of this report, the role of a number of organic compounds and inorganic ions in stimulating the germination of bacterial endospores was discussed. It was noted that the mechanism (s?) by which such agents induce germination is not known. More recent studies, however, have provided an insight into the basic nature of the biochemical transformations which accompany (or, more exactly, result in) germination and sporulation.

Foster and co-workers (12, 17, 18, 38, 39) have contributed greatly to our knowledge of the biochemical nature of sporogenesis in experiments involving "endotrophic sporulation". Briefly, this process consists of shaking vegetative cells in distilled water until sporulation occurs (usually in 12 hours); the transition from vegetative form to spore takes place in the complete absence of exogenous materials and arises as a result of intracellular rearrangements in protoplasmic constituents. During the first eight hours of shaking, the metabolic trend toward sporulation can be altered or reversed by the addition of certain agents (glucose, low pH, certain antimetabolites, etc.) but after this time the cells (then referred to as "prespores") are irreversibly committed to complete sporulation although spores are not observed until the 9-10th hour and sometimes much later. Although some objection to the conclusion that the transition is controlled exclusively by endogeneous shifts in metabolism (42) has been raised by some workers who claimed that lysis of vegetative cells occurring during the early phases of endotrophic sporulation provide exogeneous substances capable of influencing the transition, the later work of

1. The opinions expressed herein are those of the author and are not necessarily similar to the opinions of the Department of the Navy.

Perry & Foster (38) has substantiated the endogeneous nature of the process.

The intracellular changes which take place during endotrophic sporulation have been described by Foster and Perry (12). Using *B. mycoides* cells grown in the presence of $Na_2 S^{35} O_4$, the distribution of radioactivity between the hot ethanol extractable and non-extractable fractions was initially determined at various intervals during endotrophic sporulation; cells grown in this manner contain significant amounts of labelled cystine, methionine and protein, the latter being insoluble in ethanol. During the first 4 hours of shaking the extractable fraction increases sharply and the non-extractable fraction decreases. Thereafter, a reversal of this trend is observed as the ethanol-soluble radioactivity decreases to approximately its original level and the non-extractable fraction rises slightly and then levels off. Thus, during the initial phases of the transition preceding the prespore stage, the protein of the vegetative cell is rapidly degraded into low molecular weight components; following this, the concentration of the latter drops, indicating a renewal of protein synthesis, and the mature spore ultimately develops.

The fluctuations between the distribution of protein and low molecular weight degradation products are not merely successive hydrolysis and synthesis of the same materials over a period of time. The "spore protein" is a distinctly different material than the "vegetative protein", as might be suspected. Hardwick and Foster (18) have shown that comparative enzymatic activities of the vegetative cell, prespore and spore exhibit a gradual regression from relatively high activity to a completely dormant condition. Profound alterations in the protein of the cell during endotrophic sporulation are thus evident.

Since the concentration of free purines and pyrimidines also drops during the first 8 hours of shaking vegetative cells under these conditions (12), it appears that nucleic acids or, more probably, nucleoproteins are the new substances being synthesized during the early stages of sporulation. Graphic evidence of this is shown by experiments in which norleucine (a methionine antagonist) and 2, 6-diaminopurine (an adenine antimetabolite) are added to cells undergoing endotrophic sporulation. Norleucine prevents the incorporation of glutamic acid, alanine, arginine and aspartic acid into protein as

evident from the failure of these acids to show the characteristic drop in concentration during sporulation. Likewise, 2, 6-diaminopurine does not permit utilization of adenine, guanine, uracil, thymine and the above acids. These data indicate two significant features of the process: an "all or none" phenomenon occurs during incorporation of amino acids, purines and pyrimidines into high molecular weight substances; and, perhaps more important, nucleic acid and protein synthesis are intimately related, since a purine antagonist affects synthetic reactions involving amino acids.

The rationale that germination of the endospore is the reversal of sporulation seems attractive; in fact, the likelihood of such a relationship has been noted in at least one instance (35). Evidence of this is lacking, although it is certain that intracellular rearrangements of spore material are also of significance in the germinative process. Unfortunately, a study comparable to the Foster and Perry work demonstrating shifts in protein metabolism during sporulation has not been performed with the germinative process. However, a significant investigation by Fitz-James showing shifts in phosphorus distribution during germination has recently appeared and is valuable in providing an insight into intracellular events occurring during the transition (9, 10).

In the initial part of this study (9), differences between the various phosphorus (P) fractions present in spores and vegetative cells of *B. cereus* and *B. megaterium* were delineated. Comparison of the total P of spores of the two species revealed certain dissimilarities. Although both species show similar amounts of nucleic acids, *B. megaterium* spores contain twice as much total P as *B. cereus*. The excess P in the former species occurs as an acid and alkali insoluble residue associated with the spore coat. Ribonucleic acid comprises 50% of the total P of *B. cereus* spores (3-4% on a dry weight basis) but only 25% of the amount found in spores of *B. megaterium*. In general, spores contain approximately three times as much ribonucleic acid (RNA) as desoxyribonucleic acid (DNA).

Differences between vegetative cells and spores were also noted with both species. The RNA-P of vegetative cells is greater than that of spores in both organisms,* although the actual cytoplasmic

* Bennett and Williams (1) have also observed that vegetative cells contain approximately two to four times as much RNA and DNA as spores in six strains of *B. anthracis* and three strains of *B. cereus*.

concentrations of vegetative and spore RNA may not be different because of the smaller amount of cytoplasm in the latter (non-cytoplasmic materials represent a higher percentage of the mass in the spore than in the vegetative cell). The patterns of ribonuclease activity with vegetative and spore RNA are similar. The concentration of DNA-P in vegetative cells is higher than the comparable fraction in spores; this would be expected since the spores have a single nucleus and vegetative cells possess a binucleate structure. The acid and alkali insoluble residue associated with the spore coat of *B. megaterium* is very low in vegetative cells. In addition, an acid-labile phosphorus fraction (polymetaphosphate?) is much less in vegetative cells than in spores, although some doubt as to the significance of these findings was noted (8). Vegetative cells also contain a greater proportion of soluble forms of phosphorus than spores. The spore coats of *B. megaterium* are lysozyme-resistant in contrast to the cell walls of vegetative forms which are susceptible to this enzyme.

In the second portion of the Fitz-James study (10), the changes in total P, RNA-P, DNA-P, trichloracetic acid-soluble P and other fractions were carefully observed during spore germination. Data were obtained in all instances using minimal media containing a nitrogen source to enable complete germination into a mature bacillus, as described above. Under such conditions, the dry weight of the spore falls (due to inbibition of water and excretion of solids), refractility is lost and respiration is initiated during the first few minutes after inoculation into the medium. The only chemical change observed during this period is a simultaneous drop in protein-bound P and a rise in acid-soluble P, indicating that degradative reactions are predominating. No uptake of exogenous P is noted until ten minutes after inoculation. At this time, a significant rise in RNA-P occurs, and the acid-soluble P concentration and rate of respiration continue to increase, indicating the initiation of nucleic acid synthesis. Five to ten minutes later, a rise in DNA-P is also observed. At this point, a slight drop in the rate of RNA-P is noted and the spores change from an ovoid form to a cylindrical one. Subsequently, the rates of synthesis of RNA-P and DNA-P continue without alteration, being proportional to the increase in cell volume in the case of RNA-P and to growth and separation of nuclear material in the case of

DNA-P. It was suggested by Fitz-James that the RNA which is synthesized during the first 30 minutes may be "nuclear" RNA associated with chromatin synthesis and that the subsequent rise in RNA after the initial deceleration concurrent with DNA synthesis may be due to extensive build-up of "cytoplasmic" RNA.

Thus, the transition from spore to vegetative form, as measured by the distribution of intracellular phosphorus, shows the same general pattern of biochemical change as sporogenesis, i.e., initial degradation of macromolecular material followed by extensive synthesis. In both germination and sporulation, degradation and synthesis of nucleoprotein appear to be the basic process involved. In germination, the process requires the presence of inducing agents, such as L-alanine and adenosine, which initiate the "awakening" of the dormant spore by extensive hydrolytic reactions and permit the transition through "Phase One". Subsequently, in the presence of other medium ingredients (glucose, phosphate, casein digest, yeast or lactalbumin hydrolysates, etc.), nucleic acid synthesis (or perhaps more correctly, nucleoprotein synthesis) begin and the pattern of P distribution during further development of the cell is similar to if not identical with the normal vegetative growth process (*2*).

Enzymatic Activities of Endospores

The pattern of resistivity of bacterial endospores to such deleterious agents as disinfectants, dessication and heat is striking. However, an even more remarkable characteristic of these biological forms is their astonishing life span. It has been stated (*26*) that our current knowledge of the limits of this life span may not be complete since the age of some spores probably exceeds the age of microbiology as a science. Since life is characterized by a dynamic metabolic state, which ultimately exhibits the phenomenon known as "aging", the energetics of the metabolism of endospores have been of great interest to physiologists. Apparently, the relative metabolic inactivity of dormant spores (resulting in an exceedingly long life under proper conditions) is unparalleled by any other known microbiological form However, some physiological processes must be operative in the dormant spore since the complete absence of metabolic activity results in rapid death.

As indicated by Stewart and Halvorson (*47*), early demonstrations of catalytic activity in resting spores were probably due to a

failure to eliminate vegetative debris and partially germinated spores which show significant metabolic activity. More recent studies have been conducted with spore suspensions carefully prepared to eliminate such material and give more valid insight into the enzymatic complement of the endospore.

The resting endospore possesses a very low level of metabolic activity in almost all respects. However, slight alterations in the spore material produce a marked re-awakening of metabolic activity. For example, heat-shocking accelerates the rate of endogenous respiration to detectable levels (5). In the absolute sense, such spores are not truly dormant spores, even during the initial minutes of exposure to substrates since the heat treatment has obviously altered the intracellular material in some way. It is important to note, therefore, the exact condition of the spore in describing enzymatic activity attributed to such forms of life.

Perhaps the data of Hardwick and Foster (18) provide the most lucid presentation of the enzymatic regression which occurs in the transition of vegetative cell to spore. The findings are summarized in Table 2 (present author's summary). The term "prespore" has been defined above. "Inhibited prespore" refers to a cell suspension undergoing endotrophic sporulation to which glucose is added during the shaking; this serves to reverse the natural trend toward sporulation and to maintain, at least in part, a vegetative-type metabolism. The spore produced under these conditions is apparently a truly dormant form.

The findings shown in Table 2 substantiate in part the implication derived above on the basic nature of sporulation: the transition involves initial degradation of vegetative cell material followed by synthesis of spore substance. During the first eight hours of shaking in distilled water, i.e., up to the pre-spore stage, oxidative reactions are drastically reduced but reactions involving conversion of amino acids continue at a level equivalent to or greater than those in the vegetative cell. In the presence of the sporulation inhibitor, glucose, oxidative activities are maintained at a higher level than in the uninhibited prespore. No enzymatic activity is detected in the dormant, mature endospore.

Although enzymes catalyzing certain deaminative, transaminative and oxidative reactions in dormant spores could not be detected

in the above study, other workers have subsequently demonstrated significant enzymatic activities in resting endospores, i.e. proteolysis (33), heat-resistant catalase (29), amino acid racemization, (3, 47) and cleavage of nucleosides and nucleotides (30, 31).

TABLE 2

DISTRIBUTION OF ENZYMATIC ACTIVITY IN THE SPORE, PRESPORE,[1] INHIBITED PRESPORE [2] AND VEGETATIVE CELL OF BACILLUS MYCOIDES DURING ENDOTROPHIC SPORULATION [3, 4]

	Relative Distribution			
	Vegetative cell	*Inhibited prespore*	*Prespore*	*Spore (16-24*
Enzyme activity	*(0 hours)*	*(8 hours)*	*(8 hours)*	*hours)*
Catalase	1.0	0.61	2.0	0
Transaminase activity [5]	1.0	0.95 [9] (0.93-0.97)	—	0
Aerobic oxidative activity [6]	1.0	0.63 [9] (0.58-0.67)	0.14 [9] (0.13-0.15)	0
Dehydrogenase activity [7]	1.0	0.32 [9] (0.04-0.68)	0.29 [9] (0.20-0.37)	0
Deaminase activity [8]	1.0	—	1.5 [9] (1.2-2.0)	0

1, 2, 3. For explanation of these terms see text.
4. Data summarized from Hardwick and Foster (18).
5. In four different combinations of reactions involving pyruvate, alanine, glutamate aspartate, α-ketoglutarate and oxalacetate.
6. Malate, succinate, α-ketoglutarate and pyruvate as substrates.
7. Malate, succinate, glutamate, alanine and serine as substrates.
8. Serine, alanine and glutamine as substrates.
9. Average values. Numbers in parentheses indicate ranges of activities obtained with the various substrates.

The proteolytic activity of spores has been studied only superficially. Using homogenates of resting *B. megaterium* spores, degradations of gelatin, egg albumin and proteins of the spore homogenates have been demonstrated by Levinson and Sevag (33). Manganese, which stimulates germination in this species, also accelerates the rate of proteolysis.

An enzyme in spores which catalyzes the racemization of alanine has been investigated in more detail. Although alanine racemase is present in resting spores of many species and has not been shown to be directly related to the rate of spore germination (3), it is unusual in that the concentration in spores is greater than in vegetative cells. The racemase of *B. cereus* var *terminalis* is reversible, giving racemic mixtures on the addition of either the L- or D-amino acid, has an optimal pH of 8.5, is specific for alanine, and has a low affinity for substrate ($Ks = 5.8 \times 10^{-3}M$). Although pyridoxal phosphate does not influence the rate of racemase activity of intact spores, the addition of this cofactor to dialyzed spore extracts gives a threefold increase in the rate of racemization. The properties of this enzyme are similar to those of the purified alanine racemase obtained from *Strepococcus faecalis* (47).

A heat-resistant catalase present in resting spores of *B. cereus* var *terminalis* has been demonstrated by Lawrence and Halvorson (29). The enzyme is an intimate part of the spore structure and not related to contamination with vegetative debris, which frequently contains heat-labile catalase; in fact, determination of the heat resistance of catalase can be employed as a criterion of the absence of extraneous material in "clean" spore suspensions.

Until recently, no information on spore enzymes of possible significance in nucleic acid metabolism has been available. However, two recent reports by Lawrence have shown the presence of such enzymes in spores and have initiated interest in this phase of spore metabolism. Both reports concern the properties of a nucleoside and nucleotide cleavage system which, as discussed in Part I, cannot be directly correlated with the rate of germination but, nevertheless, appears to play some role therein. Cleavage of adenosine has been studied more intensively (30). Adenosine is rapidly cleaved by resting spores into adenine and ribose. Arsenate or phosphate has no effect on the rate of cleavage, indicating that the action is hydrolytic and not phosphorolytic. An exchange reaction between adenosine and hypoxanthine cannot be detected, although this is not unexpected since inosine is also cleaved by the system. The cleavage reaction is heat stable, having a high optimal temperature of about 88° C. and an optimal pH of between 5 and 6. Of paramount significance in the adenosine cleavage reaction is the failure to account for an amount

of ribose stiochiometrically equivalent to the adenine released during the reaction. Oxidation of ribose does not occur and the utilization of the pentose cannot be accounted for in this way. Although the existence of a pathway other than hydrolytic cleavage was offered by Lawrence as a possible explanation of this anomaly, the probability of such a pathway was considered unlikely.

In the second report by Lawrence (31), cleavage of other ribosides (guanosine, inosine and xanthosine) and ribotides (adenosine 5-phosphate and guanosine 5-phosphate) by dormant spores of *B. cereus* var *terminalis* was demonstrated; free purine and pyrimidine bases were detected in all instances. No evidence of the reversal of the adenosine cleavage was observed, and cleavage occurred under certain conditions inhibiting germination (low pH and high incubation temperature). It was suggested that adenosine cleavage may be the initial step in endospore germination since a correlation between the presence of a cleavage system and an adenosine requirement for germination has been observed in several species of *Bacillus*.

Respiratory studies on bacterial endospores have been of prime interest to workers in the field for some time because of the dormancy exhibited by resting spores. The earlier literature has been briefly reviewed by Crooks (5), Levinson and Sevag (32) and Stewart and Halvorson (47). Claims of high endogenous respiration reported prior to 1947 were undoubtedly due to a failure to use clean spore suspensions in many instances, although later demonstrations of significant amounts of diaphorase activity in spores (46) showed the existence of a mechanism for oxidation.

It is now known that resting spores do exhibit a detectable endogenous respiration, although the rate is so low that specialized equipment is required for accurate measurements (5). "Heat-shocking" affects significantly the level of endogenous respiration in a quantitative manner, i.e. heating anthrax spores* at 80° C. for 30 minutes will give an endogenous Q_{O2} of about 5 compared to 1.7 after "shocking" at 65° C. for 30 minutes and 0.3 without "heat-activation". The endogenous respiration in almost all instances shows an initial erratic burst of O_2 uptake which occurs over a period extending up to six hours after which the respiration is maintained at a uniform level for up to 20 hours. Under such conditions the initial

* An avirulent strain described as *B. cereus* var *anthracis*.

stage of germination (loss of refractility) is not observed, and no evidence of the nature of the endogenous substrates being utilized is available.

Significant oxidation of exogenous substrates by intact spores has, until quite recently, been invariably associated with germination of the spores. Lævinson and Sevag (32) have investigated intensively glucose oxidation by spores of *B. megaterium*. "Heat-shocked" spores were shown to germinate rapidly, the rate of germination being almost directly proportional to the rate of oxygen consumption in the presence of glucose. Of more importance, manganese plays a key role in stimulating oxidation and germination and may substitute for "heat-shocking" in initiating a rapid rate of metabolic activity. Cobalt and zinc also show some stimulatory effect on respiration, but iron, copper and high phosphate concentrations suppress oxidation. Chloride also influences strongly the rate of glucose utilization under these conditions.

In 1955, Church and Halvorson demonstrated for the first time the presence of spore enzymes of *B. cereus* var *terminalis* capable of oxidizing glucose, at least in part, through known metabolic pathways (4). Heat-activation was required to demonstrate rapid glucose oxidation by intact spores, but enzymes capable of degrading glucose are present even in spores not submitted to heat treatment, as shown by studies on spore homogenates. No evidence of germination is observed as a result of the heat-shocking process (L-alanine and adenosine absent) and the glucose degrading mechanism is a true spore characteristic. The actual mechanism of degradation involves a number of pathways, and activated spores oxidize glucose at least in part, to pyruvate. The more important glucose degradative mechanism appears not to involve the Emden-Meyerhof pathway, although some breakdown may occur through the reactions of this route. The major pathway involves dissimilation through the classical hexose monophosphate shunt, at least as far as the successive formation of glucose-6-phosphate and 6-phospho-gluconate, and through a second pathway, involving the formation of gluconic acid, 2-ketogluconate and 6-phospho-2-ketogluconate. The two pathways are interrelated through a reaction in which gluconic acid is phosphorylated to 6-phosphogluconate. Significant amounts of gluconic acid and phosphorylated intermediates accumulate during oxidation. Subsequent degradative

reactions down to pyruvate have not been identified, but it appears that an enzymatic step involving a C_5 compound is missing in the spore.* Pyruvate may be oxidized, at least in part, by the tricarboxylic acid cycle although citrate and α-ketoglutarate utilization or synthesis has not been demonstrated; the operation of this cycle as a terminal oxidative mechanism seems probable since vegetative cells of certain species of *Bacillus* oxidize pyruvate in this way.

In general, the concept that endospores are enzymatically inactive, even in the relative sense, is no longer tenable. Catalyzed reactions involving proteolysis, racemization, nucleoside and nucleotide cleavage, peroxide decomposition and glucose oxidation have been demonstrated although it is probable that the extent of some of these activities is influenced markedly by "heat-shocking" as a prelude to germination. Time-activity relationships are important in such studies and the failure to observe detectable enzymatic response may be due, in many instances, to slow rates of reaction rather than to an absence or a dormancy of available enzymes.

Discussion

Obviously, the above findings do not permit postulation of the exact sequence of metabolic events which are induced by exogenous stimulants and which ultimately culminate in the germination of the bacterial endospore. Perhaps a single mechanism for all species does not exist, since species-differences are frequently observed as noted above. However, these differences are not striking and are, perhaps, even offset by the observed similarities between genera, e.g. stimulation of germination of *Bacillus* and *Clostridium* species by L-alanine, L-phenylalanine and L-arginine (23, 24).

In reviewing all of the above findings, one is impressed by the apparent parallelism between the biochemical intricacies of germination or sporulation and those of adaptive enzyme formation. Other workers have also noted this similarity (12, 35). In both phenomena, the formation of a free amino acid pool from pre-existing protein for re-synthesis into other protein in response to an inducer can be visualized, although no direct evidence of this exists in the germinative process. (Solubilization of protein-bound phosphorus, as de-

* A possible relation may exist between this observation and the failure to account for the ribose resulting from cleavage of adenosine (30).

scribed by Fitz-James, is no assurance of the existence of a free amino acid pool.) In both processes, amino acids are observed to vary widely in the capacity to elicit either an adaptive enzymatic response or induce germination. In both phenomena, autocatalytic amounts of inducer may "trigger off" striking intracellular degradative and synthetic actions. Also, similarities in a characteristic competition for nitrogenous substances are evident in both adaptation and sporulation; this is graphically shown in the latter by the ability of glucose to alter the natural trend toward sporulation and maintain a vegetative-type metabolism during endotrophic sporulation. Finally, it is apparent that both adaptive enzyme formation (36, 37) and germination (10) are influenced strongly, if not completely controlled, by the interrelation of cellular nucleic acid and protein metabolism. Along this line, Fitz-James has observed a significant level of a polymerized metaphosphate in *B. cereus;* such material is believed to exist as nucleic acid-bound phosphorus in micro-organisms and may serve as a source of energy required for peptide bond synthesis on hydrolysis of such nucleic acids (7).

The relationship between the energy requirements of sporulation and germination has not yet been established. Sporulation can occur without addition of exogenous substances; on the other hand, germination requires at least autocatalytic amounts of inducer. The failure of spores to germinate endogenously has been shown by Crooks (5), who shook *B. cereus* var *anthracis* spores in distilled water for 20 hours without indication of germination. In spite of the failure to observe significant endogenous germination, some workers believe that development of the endospore to the initial phase of germination does not require a source of exogenous energy. In the evidence to support this contention (16), specific inhibitors capable of blocking energy-yielding and associated reactions (oxidation, glycolysis, phosphorylation, and uncoupling of energy-rich phosphate bonds) were shown to have no significant effect on the rate of germination of *B. subtilis* spores as measured by the loss in refractility. Certain of these inhibitors (cyanide, arsenite, monoiodoacetate and 2, 4-dinitrophenol) were effective in preventing growth of the germinated vegetative cells, as measured by the appearance of pellicular growth. It was suggested that the initial phase of germination differs, therefore, from the remainder of the process, i.e. germination

and subsequent growth, in that no exogenous energy is required by the former. Unfortunately, the findings presented by these workers do not represent conclusive evidence of energy requirements during germination since other factors, such as the permeability of spores to the inhibitors, may have contributed to the findings. Nevertheless, the data are a valuable initial step in the solution of the problem.

The role of "heat-activation" in accelerating germination of spores in the presence of an inducer is interesting. Undoubtedly, the preheating alters profoundly the metabolic processes of the dormant spore although no detectable cytological change is observed. In relation to this, Swartz et al. (48) have recently elucidated the properties of two "heat-activated" enzymes from *Proteus vulgaris:* a nucleotide pyrophosphatase and a 5′ nucleosidase. The "heat-activation" process acts by destroying specific intracellular protein inhibitors of enzymatic reactions; the inhibitors prevent the action of the enzymes in the natural cellular state. Other protein inhibitors of enzymatic reactions were detected in other species, including one inhibiting an enzyme in *B. subtilis* which splits diphosphopyridine nucleotide, yielding adenosine ribose diphosphate and free nicotinamide. Possibly, these observations are related to the phenomenon of heat-activation of endospores.

Another question of significance in problems involving spore metabolism is the role of dipicolinic acid, which occurs in rather large quantities in endospores. It has been shown that 2, 6-diaminopimelic acid (DPA) is converted into dipicolinic acid in *B. mycoides* and that the pattern of sporogenesis in this species parallels the rate of synthesis of dipicolinic acid (39). However, the route of conversion of DPA to picolinic acid has not been conclusively demonstrated. Hypothetically, two mechanisms may be involved: either direct formation of picolinic acid by ring closure of DPA, or initial degradation of DPA to such compounds as amino acids and conversion of the latter to dipicolinic acid. During endotrophic sporulation in the presence of C^{14} tagged DPA, labelled amino acids and dipicolinic acid are isolated, but the latter contains more radioactivity (39). Lysine is not radioactive under these conditions, indicating that a DPA→ lysine conversion similar to that encountered in *E. coli* may not be operative. Although dilution by endogenous substrates and the question of DPA permeability (8) make evaluation of such findings

difficult, preliminary evidence for an implication of DPA and dipicolinic acid in the amino acid metabolism of endospores may be suggested by these findings. Similar relationships have been postulated for *E. coli* (8).

Although the numerous reports discussed above appear both enlightening and confusing, it is apparent that interest in the basic physical and chemical forces of sporulation and germination is accelerating. New findings of a broad biochemical nature are rapidly appearing and many of these bear a direct relationship to the spore problem. The recent description of an enzyme catalyzing the synthesis of substances similar to if not identical with ribonucleic acid from purines and other RNA degradation products and involving phosphorylations analogous to polysaccharide synthesis is an example of such findings (13). Application of such findings to studies on the mechanisms of germination and sporulation should contribute greatly to the elucidation of the basic reactions involved in the transitions.

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INHIBITING CANCER IN ANIMALS. VII.

By John R. Sampey *

THE present study brings the number of articles reviewed on current animal experiments with anticancer agents to more than one thousand (160). It shows increased interest in such organic nitrogen compounds as the phosphoramides, 6-mercaptopurine, methylformamide, amino acids, and nitrogen mustards. Estrogens, cortisone and the adrenals dominate the hormone field, while folic acid antagonists, antibiotics, tissue extracts and diet are the leading miscellaneous agents tested for carcinostatic action.

I. Inorganic Chemicals

Thorotrast. Small doses of ThO_2 caused significant decreases in mouse ascites tumor, but larger doses increased the rate of growth (14). This oxide or Fe_2O_3 decreased the incidence of DAB and 3'methyl-DAB tumors in rats (35). Bernard, et al. (18, 19), however, reported no effect on Walker carcinoma 256 or from metastases from the same by administration of ThO_2 in rats.

Radioiodine and Radiophosphorus. I^{131} derivatives of Nile blue 2B prolonged the lives of mice with fibrosarcoma transplants or spontaneous breast carcinoma (172). I^{131} destroyed carcinoma thyroid implants in rats (63). Single large injections of P^{32} made mouse breast tumors necrotic or liquefy (82).

Salts. Potassium cyanide protected mice from lethal doses of x-rays (21, 22, 23). Sodium chloride decreased the mortality of mice exposed to x-rays (55), and sodium cyanide and sodium nitride also reduced the death rate of mice from x-irradiation (98, 99). Sodium salicylate acted similarly if given before exposure, but this salt shortened the lives of mice when given after exposure (25). Lambert, et al. (122), reported that $BaCl_2$ and LiCl hastened the death of rats receiving lethal doses of x-rays, while $HgCl_2$ lengthened the life span. Field, et al. (59), prolonged the lives of mice having leukemia L4946

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or Sarcoma-180 by administration of salts of heavy metals, e.g., Cu, Cd, Pb.

II. Organic Nitrogen Compounds

Nitrogen Mustards. Stock (179), found HN2 very effective against Flexner-Jobling carcinoma, Walker carcinoma and S-39. With Tarnowski (189) he reported RC mammary carcinoma was inhibited only by nitrogen mustard. Clarke (34) inhibited S-180 with HN2, and Skipper and Thomson (170) increased the life span of mice in four lines of L1210 leukemia with HN2. Burchenal, et al. (29), also found nitrogen mustard effective against mouse transplanted leukemia. Suguire (184) reported HN2 effective against adenocarcinoma E0771, Carcinoma 1025, Walker carcinoma and Flexner-Jobling carcinoma. The same investigator noted that nitrogen mustard and its oxide markedly inhibited Ehrlich ascites carcinoma, Krebs 2 and S-180 in mice (182). Creech, et al. (37), recorded that HN2 and colchicine prolonged the lives of mice with Ehrlich ascites tumor longer than any other agent. Ross (157) inhibited Walker tumor with a series of nitrogen mustard compounds, and Trams and Klopp (191) induced remissions in two transplantable tumors with HN2. Dagg, et al. (47), inhibited human tumor transplants in the chick embryo with HN2. Bessis, et al. (20), reported this agent was without effect on fowl leukemia, and Hirono (100, 101) noted resistance of Yoshida sarcoma to HN2, its oxide, and HN3 in rats.

6-MP. 6-Mercaptopurine inhibited Ehrlich ascites carcinoma (182, 37) Krebs K2 (182), S-180 (32, 33, 34, 169), adenocarcinoma 755 (71, 169), mouse leukemia L1210 (77, 126, 169), RC mammary adenocarcinoma (188), glioma 26 (71), Brown-Pearce carcinoma (71), and transplanted brain tumor in mice (70).

Methylformamide. N-Methylformamide has shown carcinostatic action against S-180 (34, 171), Ehrlich ascites (37, 67), breast adenocarcinoma 755 (71), L1210 (70, 171), adenocarcinoma E0771 (171), carcinoma 1025 (184), and RC mammary adenocarcinoma (188).

Azaguanine. 8-Azaguanine retarded sarcoma T241 (184), Ehrlich ascites (37), mammary adenocarcinoma 755 (71), Brown-Pearce carcinoma (71), mouse leukemia L1210 (126), adenocar-

cinoma E0771 (179, 184), Flexner-Jobling carcinoma (184), carcinoma 1025 (184), Mecca lymphosarcoma (184), and a transplanted brain tumor (70). Sokoloff, et al. (174), reported azaguanine had little effect on August rat carcinoma.

Ethyleneimines. Triethylene melamine, TEM, induced regression in Flexner-Jobling carcinoma (43, 44, 45, 179, 183), S-231 (45), S-39 (179), and S-180 (177, 179), and Walker carcinoma (179). Yoshida sarcoma was resistant to TEM (101). Triethylene phosphoramide, TEPA, and derivatives retarded lymphomas in dogs (5), S-231 (44), mouse mammary 72-J tumor (54), mouse brain tumor 8110 (54), transplanted breast adenocarcinoma (163), myelocytic chloroleukemia (168), Jensen sarcoma (183), Krebs K2 (182), S-R39 (183), and mouse ascites (182).

Mercaptoethylamine. β-Mercaptoethylamine increased the survival of mice (12, 13, 103), and guinea pigs (89) exposed to radiation.

Thioureas. Thiourea lowered the mortality of mice given heavy x-irradiation (132, 142), but Haley, et al. (91), found no protection for rats given non-lethal doses of the drug. Naphthylthiourea plus gum arabic gave rats protection against lethal doses of x-rays (123). Thiourea inhibited the growth of transplanted fusiform sarcoma of the rat (46).

Pyrimidines. Thymine and dihydrothymine reduced the incidence of spontaneous lung tumors in mice (60). The antimetabolite, 50-197, alone or with ethionine inhibited carcinoma 755 in mice (167).

Pyridines. Pyridine substituted thiosemicarbazone increased the life span of mice with L1210 leukemia (27).

Alloxan. Alloxan increased the survival of mice with Ehrlich ascites carcinoma (112, 113), and it reduced the size of Walker carcinoma 256 and hepatomas in rats (80).

Purines. 2,6-Diaminopurine inhibited mouse leukemia L1210 (126) and S-39 (179).

Quinolines. Hughes, et al. (105), reported complete regression of rat lymphoma No. 8 following administration of styryl quinolines.

Urethan. Urethan prolonged the lives of mice with leukemia L82 (28), but the compound had no effect on fowl leukemia (20), or mouse leukemia L1210 (171).

Aminodiphenyl. 4-Acetylaminodiphenyl failed to protect rats from the carcinogen, AAF (158).

Amino Acids. Sulfur amino acids have been employed to protect rabbits (153), guinea pigs (64), mice and rats (40, 41, 42, 75, 125) from x-rays. Ethionine induced regression of breast adenocarcinoma 755 and glioma 26 in mice (71). Azaserine inhibited Mecca lymphosarcoma, Flexner-Jobling carcinoma, Walker carcinoma and adenocarcinoma 755 (184, 185). D-Form of tryptophane and of lysine retarded breast tumors in mice (72). Yoshida sarcoma was retarded by n-bis-β-chloroethylalanine (109). Shapiro and Wolkonsky (167) were unable to confirm the inhibition of mouse breast adenocarcinoma 755 by ethionine alone. Hammer, et al. (94), noted negative results with zinc salts of amino acids in mice with implanted tumors.

III. Hormones

Estrogens. Estradiol inhibited adenomas in castrated rats (6), and it retarded luteoma in guinea pigs (107). Estradiol benzoate, benzestrol and other sex hormones protected mice from x-rays (84, 151). Estrogens modified the growth of breast cancer in mice (198). Stilbestrol did not alter AAF induced hepatomas in rats (128).

Androgens. Testosterone inhibited lymphoma formation in irradiated mice (68). Androgens modified the growth of breast cancer in mice (198).

Adrenal Hormones. ACTH and cortical extracts protected adrenalectomized rats from x-rays (24, 115). Cortisone inhibited the growth of methylcholanthrene induced sarcoma in the cheek pouch of hamsters (130), DMBA induced tumors in mice (156), transplanted breast cancer in mice (198), and bone tumors and lymphosarcoma in mice (178). Hydrocortisone and ACTH retarded transplanted breast adenocarcinoma in mice (163, 175). ACTH and cortisone produced marked decrease in the growth of DBA induced breast cancer in mice when given with growth hormone (141), but both hormones failed to increase the survival of irradiated mice (173), or the growth of AAF induced hepatomas in rats (128). Small doses of cortisol given with NH_4Cl caused pronounced retardation of Walker 256 tumors (166). Rats with lead shields around the adrenal glands survived longer after x-irradiation (50). Desoxycorticosterone in-

hibited rat adenomas (6), and protected rats from x-irradiation (51, 123). Adrenalectomy did not modify tumor regression in mice from x-rays (104), or influence the sensitivity of mice to x-rays (181). Transplantation of adrenals to the spleen of rats had no effect on AAF tumors (154). Transplanted sarcoma in rats were inhibited by adrenalin (131).

Growth Hormone. Growth hormone, STH, protected mice against sublethal doses of x-rays (165, 176), but it did not protect them against large doses of irradiation (81, 165). STH inhibited breast adenocarcinoma in mice (175).

Thyroid Hormone. Mice given D,L-thyroxin had less spontaneous leukemia than controls (83), but thyroid deficient animals survived longer after injections of lymphoid leukemia. Depression of thyroid function protected rats from estrogen induced pituitary tumors (73). Thyroid deficient mice survived longer after injections of lymphoid leukemia (146).

Thymus. Thymectomy reduced the incidence of methylcholanthrene induced leukemia in mice (119). A combination of thymic extract and β-mercaptoethylamine protected guinea pigs from x-rays (89).

Pineal. Pineal gland extracts inhibited adenocarcinoma in rodents (56).

Hypophysectomy. Hypophysectomized mice had radiation induced lymphoid tumors (147), and hypophysectomy also caused regression of ovarian grafts in rats (26).

IV. Miscellaneous

Folic Acid Antagonists. Amethopterin inhibited leukemia L1210 in mice (77, 78, 126), and erythroblastose in fowls (20). Amethopterin with 6-MP was more effective than either drug alone against L1210 (169), and with citrovorum factor, CF, it was more effective against leukemia in mice than either drug alone (79, 118). Aminopterin inhibited Yoshida sarcoma (8), and lymphoid tumors in fowls (31). Amethopterin showed a carcinostatic action against S-180 in mice (34, 179), Ehrlich ascites (37), S-39 (179), Walker carcinoma 256 (179), and lymphoid tumors in chicks (31). Teropterin inhibited

transplanted carcinoma in animals (192). Folic acid failed to influence lethal radiation in swine (39).

Synkavit. 2-Methyl-1,4-naphthohydroquinone diphosphate, Synkavit, had no effect on eight classes of tumors in mice and rabbits, including S-180, Brown-Pearce carcinoma, and adenocarcinoma (69). Jolles and Laws (114) reported this vitamin K substitute gave a slight increase in tolerance of x-rays in rabbits, but in rats the opposite effect was noted. Dittrich and Schmermund (49) found this agent increased resistance of Ehrlich carcinoma to x-rays.

Antibiotics. Aureomycin (124, 137), streptomycin (103, 137), and penicillin (137) prolonged the lives of rodents receiving lethal doses of x-rays, but Gordon, et al. (81), reported streptomycin failed to protect mice from total body irradiation. Aureomycin and penicillin have been used to suppress bacterial syntheses of l-acids and thus retard the growth of carcinoma and ascites hepatoma (96, 195). Puromycin showed a carcinostatic action on breast adenocarcinoma in mice (92, 93, 193) and glioblastoma in the chick embryo (193).

Cell and Tissue Extracts. Lives of irradiated animals were prolonged by water insoluble liver residues (58), by bone sarcoma (84), and by plural exudate (180). Ascites tumor was inhibited by antiseria from rabbits (36), and anti-Ehrlich globulin complement (61). Breast tumors were delayed by extracts of amphibian skin and gills (97), plant extracts (138), and endotoxin of *T. Cruzi* (143). Red cell stromata caused resistance to sarcoma 49 implants in mice (15, 16). Implants of tumor tissue retarded the growth of fibroma grafts in rats (62), and human tumors retarded the growth of rat sarcoma when both were placed in the yolk sac of embryonated chicken eggs (200).

Diet. Liver tumors were inhibited by a high riboflavin diet (57), a high protein diet (127, 152), and a high fat diet (139). Feeding liver retarded AAF and DAB tumors (145). Ehrlich ascites carcinoma grew less on a fat diet (2). Rats left on an iodine deficient diet and given thiouracil showed a significant decrease in lymphogeneous leukemia (83). Yeast or synthetic vitamin B in the diet of rats reduced DMBA carcinogenesis (164). Female mice made obese developed breast tumors earlier, but when subsequently reduced to the weight of the controls, they showed a slower rate of tumor incidence (197).

Viruses. The cancercidal properties of Louping-ill, West Nile, Illeus and other viruses have been studied (121, 133, 149, 190).

Trypan Blue. This dye strongly inhibited DAB induced hepatomas in rats (110, 111, 161).

Nitromin. Ascites hepatomas in rats (120), and MC induced skin carcinomas in mice (148) were retarded by nitromin, but DAB induced hepatomas in rats were not affected (116).

Bacteria. A vaccin of bacteria isolated from an ulcerating tumor in mice inhibited breast adenocarcinoma when injected with ACTH (10). A combination of yeast extract and extract of *glycyrrhiza globra per os.* retarded Brown-Pearce tumors in rabbits and carcinomas in mice (11). Mixed bacterial toxins from streptococci and erysipelas caused 60% regression of S-37 in mice (95).

Enzymes. Ribonuclease injection prolonged the lives of animals with Ehrlich, Krebs and Walker carcinomas (129). Luhrs and Willig (134) reported hyaluronidase was without effect on ascites tumor or S-37 in mice.

Podophyllotoxin. This agent damaged sarcomas in rodents (3, 196).

Polysaccharides. These compounds inhibited Ehrlich ascites carcinoma (186) and S-37 (17) in mice.

Alcohols. Propanols, butanols, glycerine and other alcohols protected mice from x-rays (52, 159, 199).

Dimethanesulfonyloxybutane. This chemical inhibited transplanted brain tumors (70), glioma 26 and Brown-Pearce carcinoma and adenocarcinoma 755 in mice (71).

Citrovorum Factor. C. F. plus aminopterin was effective against mouse leukemia (79, 118).

Alkaloids. Demecolcin (Substance F), a new alkaloid from colchicum autumnale, retarded adenocarcinoma E 0771 in mice (162), while the latex of *Fiens Carcia L.*, an alkaloidlike substance delayed S-180, adenocarcinoma E 0771 and other neoplasms (194).

Colchicines. Colchicine was effective against Ehrlich ascites tumor in mice (37), Brown-Pearce carcinoma (71). Thiocolchicine

was 2.5 times more potent against Yoshida sarcoma than colchicine (1).

Bio-Flavonoid. Bio-flavonoid extracted from citrus fruits increased the tolerance of rats to contact radiation (7).

Cacodylic Acid. This chemical had no effect on Yoshida sarcoma (9).

Periston and Rutin. Neither of these agents reduced the lethality of x-irradiation in rodents (30, 38).

Quercetrin. Fusiform sarcoma was not affected by this agent (46).

Fluorocitrate. This chemical exerted a carcinostatic effect on adenocarcinoma 755 in mice (48).

Hexamethylolmelamine. Mouse ascites tumor was retarded by this agent (53).

Diethylethoxymethylenemalonate. This compound inhibited carcinoma 755 in mice (65).

Methylation. N-Methylation enhanced the cancercidal action of formic hydrazide but not that of acetic hydrazide (66).

Cytochrome C. This agent had no effect on mouse adenocarcinoma (74).

Pitressin. Pitressin decreased S-180 cells in the peritoneal fluid of mice (76).

Ketones. p-Hydroxypropiophenone inhibited 3′-Methyl-DAB carcinogenesis in rats (155), and the p-amino derivative of this ketone afforded rats protection against x-rays (85).

Furfural. Furfural reduced DAB tumors in rats (108).

Shock and Stress. Induction of the state of shock by adenosine triphosphate had no effect on the growth of transplanted sarcoma in rats (86). Temperature stress inhibited 891 melanoma and S-91A melanoma in mice (106).

Sulfur Mustard. Bis-(β-chloroethylthio)-ethane was effective in inhibiting a variety of neoplasms in rodents (88).

Antihistamines. Several of these drugs increased the mortality of x-irradiated guinea pigs (90).

Mustard Compress. Ground mustard compresses retarded Jensen sarcoma and combined with x-rays they showed a synergistic effect (102).

Cholines. Ethanolamine markedly reduced azo dye liver tumors (117).

Gum Arabic. This agent shortened the lives of some rats exposed to x-rays, while others lived longer (123).

Milk Factor. A factor transmitted by milk from foster nursing lengthened the lives and reduced the incidence of mouse leukemia (135, 136).

Dithiophosphonate. Sodium ethane dithiophosphonate protected mice from x-rays (142).

Allergic Inflammation. Fibrosarcoma 5621 and S-I in mice were unaffected by allergic inflammation produced by injections of crystalline ovalbumin (144).

Flavonoids. These agents protected mice from x-rays (150).

Myleran. Ascites tumor completely regressed in more than half the rats receiving this drug (187).

Chlortetracyline. Harderian gland carcinoma and ovarian adenocarcinoma in rats regressed with this agent (191).

Histones. Histones delayed the growth of sarcoma in mice (201).

Ethylenediamine Tetra-acetic acid. Walker 256 tumors in rats were inhibited by this agent (140).

Dibenzanthracene. This hydrocarbon inhibited sarcoma transplants (87).

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